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Phosphorylation differences among proteins of bloodstream developmental stages of *Trypanosoma brucei brucei*

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Early in an infection the bloodstream forms of the African trypanosome *Trypanosoma brucei brucei* are long, slender and rapidly dividing. Later, non-dividing, short, stumpy forms may be found. In this report we describe biochemical differences between the two parasite populations in the phosphorylation of their proteins *in vitro*. Compared with the slender populations, the non-dividing stumpy forms of the parasites exhibit decreased phosphorylation of an 80 kDa protein and enhanced phosphorylation of 37 kDa and 42 kDa proteins (pp37 and pp42). These changes occurred regardless of whether the stumpy trypanosomes were generated naturally during the course of the infection or induced by difluoromethylornithine treatment. The phosphorylation of pp37 and pp42 occurs on serine and threonine residues and is totally dependent upon the presence of Mn^{2+} or Mg^{2+}. However, excess Mn^{2+} or Mg^{2+} inhibits phosphorylation. Maximal phosphorylation of pp42 occurs with 1 mM-Mn^{2+} or 10 mM-Mg^{2+}, whereas that of pp37 occurs with 50 mM-Mn^{2+} or greater than 100 mM-Mg^{2+}. The phosphorylation of pp37 is greatly enhanced by KCl, whereas that of pp42 is only slightly increased by this salt. Ca^{2+}, calmodulin, phospholipids and cyclic AMP have no discernible effect upon the phosphorylation of pp42 or pp37 *in vitro*, whereas heparin, suramin, polylysine, polyarginine and polyamines all inhibit phosphorylation. Thus the enzymes that phosphorylate pp42 and pp37 have properties similar to, but distinct from, those of mammalian casein kinase II. Since the casein-kinase-like activity is higher in the slender than in the stumpy forms, the enhanced phosphorylation of pp42 and pp37 in the non-dividing parasites is probably a result of the enhanced synthesis of these acidic proteins.

INTRODUCTION

During the life cycle of the haemoprotozoan parasite *Trypanosoma brucei* the parasites proceed through several morphologically distinct stages in both the mammalian host and the tsetse fly vector. These changes are accompanied by alterations in the biochemistry of the parasite (Mbawa *et al.*, 1991, and references therein) and are a reflection of the parasite's adaptation to the cyclical variation in its environment. In the bloodstream of the mammalian host, *T. brucei* differentiates, via intermediate forms, from rapidly dividing slender forms into non-dividing stumpy parasites. The bloodstream forms of the parasite are completely dependent on glycolysis for energy, because their tricarboxylic acid cycle is not functional (Flynn & Bowman, 1973; Bowman & Flynn, 1976; Fairlamb & Opperdoes, 1986). However, in the stumpy trypanosomes there is evidence for the partial development of the mitochondrion (Vickerman, 1965, 1970) as well as for activation of some tricarboxylic acid-cycle enzymes such as proline and α-oxoglutarate oxidases (reviewed in Vickerman, 1985) as well as *cis*-aconitase (Aboagye-Kwarteng, 1983). This partial mitochondrial development in the stumpy trypanosomes may represent a pre-adaptation for the insect midgut stage when the mitochondrion becomes fully developed and the tricarboxylic acid cycle becomes fully functional (Vickerman, 1965).

Evidence to support this hypothesis was provided by Wijers & Willett (1960), who showed that the long slender bloodstream forms are unable to differentiate in the gut of the tsetse fly. In contrast, the intermediate and stumpy form parasites are able to complete the activation of their mitochondrion (Vickerman, 1965), renew DNA synthesis (Shapiro *et al.*, 1984) and transform into the proliferating tsetse midgut (procyclic) trypanosomes. Thus differentiation from long slender to short stumpy forms is important to the life cycle and survival of the parasite. In order to understand the life cycle, the factors which control the differentiation of the trypanosomes through the various stages of the cycle need to be identified and characterized.

In recent years the central role of protein phosphorylation in signal transduction, cellular transformation and differentiation in eukaryotic systems has become well established, and complex networks of protein phosphorylation/dephosphorylation have been identified. Such networks are important mechanisms by which cellular processes are regulated (reviewed in Cohen, 1982; Nairn *et al.*, 1985; Krebs, 1985; Edelman *et al.*, 1987; Shenolikar, 1988). For example, protein phosphorylation plays an important role in cellular transformation by several oncogenes (Collett *et al.*, 1980; Hunter & Sefton, 1980; Hunter & Cooper, 1985) and in the cellular effects of several growth factors (Hunter & Cooper, 1981; Ek *et al.*, 1982; Kasuga *et al.*, 1982; White & Kahn, 1986). Studies in yeast have established that protein phosphorylation also plays an important role in control of the cell cycle. In *Schizosaccharomyces pombe* the cell cycle control gene, *cdc2*, which is required both for commitment to a new division cycle and control of mitosis, encodes a phosphoprotein of molecular mass 34 kDa ($p34^{cdc2}$) which has protein kinase activity (Nurse & Thuriaux, 1980; Nurse & Bisset, 1981; Beach *et al.*, 1982; Simanis & Nurse, 1986). When cells are arrested before 'start' (by nitrogen deprivation) $p34^{cdc2}$ becomes dephosphorylated and it loses its protein kinase activity. This protein kinase activity is regained, after a lag phase, upon refeeding the cells with a source of nitrogen. This observation is consistent with the notion that $p34^{cdc2}$ phosphorylation and activity are required for the cell to progress through the cell cycle (Lee & Nurse, 1986; Lee *et al.*, 1988). Homologues of the *S. pombe* $p34^{cdc2}$ have been identified

Abbreviations used: DFMO, difluoromethylornithine; DTT, dithiothreitol; TFA, trifluoroacetic acid.

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in human cells (Lee & Nurse, 1987) as well as in other eukaryotic species such as starfish, clam, *Xenopus*, rat and mouse (Draetta *et al.*, 1987, 1988; Arion *et al.*, 1988; Draetta & Beach, 1988; Dunphy *et al.*, 1988; Gautier *et al.*, 1988; Labbe *et al.*, 1988; Lee *et al.*, 1988).

Our objective in this study was to determine whether changes in protein phosphorylation may occur between bloodstream forms of the rapidly dividing slender and non-dividing stumpy *T. brucei*. We report here the identification and characterization of two differentially regulated phosphorylatable proteins in the bloodstream forms of *T. brucei*.

MATERIALS AND METHODS

Materials

[γ-^{32}P]ATP was obtained from Amersham, DEAE-cellulose (DE-53) from Whatman Biosystems Ltd. and Percoll from Pharmacia Fine Chemicals (Uppsala, Sweden). Difluoromethylornithine (DFMO) was kindly donated by Dr. Cyrus Bacchi, Haskins Laboratories and Biology Department, Pace University, NY, U.S.A. All solvents used for h.p.l.c. were h.p.l.c. grade and were purchased from either Pierce Chemical Co. or Merck Chemical Co. All other reagents used were at least of Analar grade.

Preparation of trypanosome lysates

T. b. brucei ILTat 1.1 (Young, 1985), grown in adult lethally irradiated (600 rad) Sprague–Dawley rats, was used in this study. The percentages of long slender and short stumpy form trypanosomes on different days after infection were determined according to Ormerod *et al.* (1963). The infections produced were sufficiently synchronous to allow 100% long slender and greater than 95% short stumpy trypanosome populations to be isolated at 4 and 7 days after infection respectively. When the desired stage of parasitaemia was attained, the trypanosomes were purified from blood components by isopycnic Percoll gradient centrifugation (Grab & Bwayo, 1982) followed by DEAE-cellulose chromatography (Lanham & Godfrey, 1970). DFMO-treated trypanosomes were obtained, as described by Bacchi *et al.* (1983), by replacing the normal drinking water of 4-day-infected rats with a 2% solution of DFMO.

Purified trypanosomes were suspended at a concentration of 5×10^9/ml in 25 mM-Hepes/250 mM-sucrose/2 mM-EDTA/10 mM-EGTA/2 mM-dithiothreitol (DTT), pH 7.0, with 40 μg/ml of each of the proteinase inhibitors leupeptin, antipain and E-64, and disrupted in a French Pressure Cell disruptor at a pressure setting of 1.73×10^7 Pa. The homogenate was centrifuged in a Beckman 18-80M ultracentrifuge (100000 ***g***, 90 min) to give a cytosolic fraction and a pellet. All fractions were stored at -70 °C until used.

Protein phosphorylation reactions

Reaction mixtures (0.15 ml) contained 50 mM-Tris/HCl buffer, pH 7.2, containing 10 mM-$MgCl_2$, 150 mM-KCl, 150 μg of trypanosome cytosolic extract (enzyme and substrate source), 5 μM-ATP and 10 μCi of [γ-^{32}P]ATP (10 Ci/mmol, Amersham). In some experiments, ATP and [γ-^{32}P]ATP were replaced with GTP and [γ-^{32}P]GTP. In experiments to determine the substrate specificity of the trypanosome protein kinases, the amount of cytosolic extract (as enzyme source) used was 10 μg, and other proteins such as dephosphorylated casein, phosvitin, histones or heat-inactivated slender and stumpy trypanosome cytosolic extracts were added to the reaction mixtures at final concentrations of 1 mg/ml. Reactions, performed on ice, were started by the addition of trypanosome extract and stopped after 10 min by adding 50 μl of 4 × SDS sample buffer [0.2 M-Tris/HCl, pH 6.8, 8% (w/v) SDS, 40% (v/v) glycerol, 4% (v/v) 2-mercaptoethanol and 0.5% (w/v) Bromophenol Blue], followed by immediate boiling for 5 min in a water bath.

Electrophoreses

Phosphorylated proteins were separated by discontinuous SDS/PAGE according to Laemmli (1970) using 7.5–15% (w/v) polyacrylamide separation gels. The gels were stained with Coomassie Blue, destained and dried. Radioactive bands were detected by exposure of the gels to X-ray film (Fuji RX) at -70 °C in the presence of an intensifying screen. Quantitative measurements of phosphate incorporation into individual bands of interest were made by scintillation counting in Aquasol (New England Nuclear) of regions excised from the gel. Regions of the gel with no visible bands upon exposure to film were also excised and used as background controls. Two-dimensional PAGE was performed as described by O'Farrell (1975).

Proteins were electroeluted from gel strips in a solution containing 50 mM-NH_4HCO_3, 1 mM-DTT and 0.1% (w/v) SDS using an electroeluter–concentrator (CBS Scientific Co.). Greater than 95% elution of the phosphoproteins was achieved by applying a current of 12 mA/cell for 18 h. Excess SDS was removed from the phosphoproteins by passing the protein solutions through an Extracti-Gel D column (Pierce Chemical Co.).

Isoelectric focusing of electroeluted ^{32}P-labelled phosphoproteins was performed as described by Ui (1971), using a 110 ml capacity LKB 8100 electrofocusing column. Electrophoresis was run at 1600 V for 18 h in the presence of 1.5% carrier ampholytes. Fractions of 1 ml were collected and radioactivity of the fractions was determined by liquid scintillation counting of 10 μl samples.

Peptide mapping

Phosphoprotein bands in SDS/polyacrylamide gels were excised from the wet gel after identification by autoradiography. The gel pieces were washed with distilled water for 30 min before incubation for 18–24 h at 37 °C in 1 ml of 100 mM-NH_4HCO_3, pH 7.9, containing 50 μg of trypsin/ml [treated with Tos-Phe-CH_2Cl (L-tosylamido-2-phenylethyl chloromethyl ketone; 'TPCK')]. After incubation the gel pieces were removed and the supernatant was lyophilized and analysed by SDS/PAGE and reversed-phase h.p.l.c. Before h.p.l.c. analysis the lyophilized samples were dissolved in 1% trifluoroacetic acid (TFA; Pierce Chemical Co.), relyophilized and redissolved in 0.1% TFA. Samples were applied to an Ultrasphere ODS column (4.6 mm × 25 cm) which had been pre-equilibrated with 0.1% TFA. After washing isocratically for 5 min at a flow rate of 1 ml/min, peptides were eluted with sequential linear gradients of acetonitrile in 0.1% TFA: 0–60% over 60 min, followed by 60–80% over 5 min. Absorbance was monitored at 214 nm and fractions (1 ml) were analysed for radioactivity by liquid scintillation counting.

Identification of phosphoamino acids

When proteins were subjected to alkaline hydrolysis, the procedure described by Martensen (1984) was employed. Otherwise, lyophilized electroeluted phosphoproteins were hydrolysed (110 °C, 1 h) in 0.5 ml of 6 M-HCl as described by Cooper *et al.* (1983). The hydrolysates were dried in a stream of dry N_2 and redissolved in 10 μl of a solution containing 2.5 mg of each of authentic phosphoserine, phosphothreonine and phosphotyrosine (Sigma Chemical Co)/ml. Phosphoamino acids were resolved by two-dimensional electrophoresis (at 5 °C) on thin layer cellulose plates (Whatman microcrystalline) as described by Manai & Cozzone (1982) using an LKB 2117 Multiphor II horizontal electrophoresis unit. In the first dimension, electro-

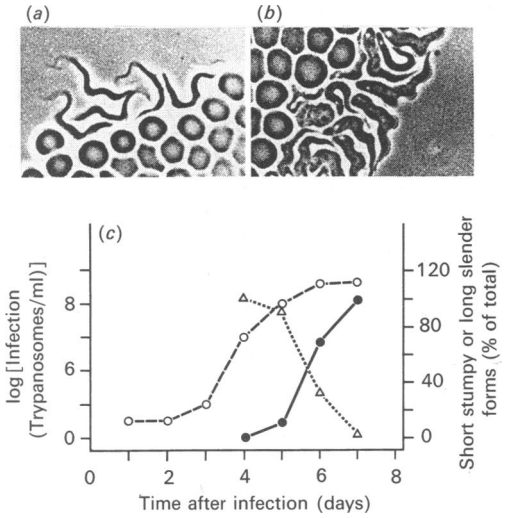

Fig. 1. Phase-contrast microscopy of bloodstream forms *T. b. brucei* (*a* and *b*), and graph showing the course of *T. b. brucei* ILTat 1.1 infection in the blood of a rat (*c*)

Tail blood from rats was examined 4–7 days after infection, on agar slides under oil immersion, by phase-contrast microscopy. (*a*) Long slender trypanosomes (4 days post-infection); (*b*) short stumpy trypanosomes (7 days after infection); (*c*) the course of infection in rat blood over 7 days, showing the change in the proportions of long slender parasites (△) and stumpy form trypanosomes (●) in relation to the total number of parasites/ml of peripheral blood (○).

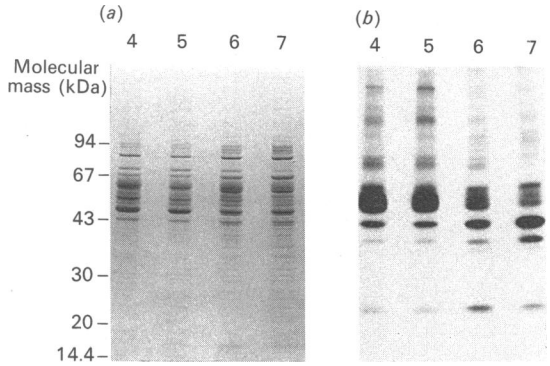

Fig. 2. Phosphoprotein profiles of bloodstream *T. b. brucei*

Cytosolic extracts of trypanosomes isolated on different days after infection, as indicated above the lanes, were phosphorylated *in vitro* with [γ-^{32}P]ATP and separated by SDS/PAGE on a 7.5%–15% (w/v) polyacrylamide gradient gel, stained with Coomassie Blue (*a*) and autoradiographed (*b*). Numbers on the left indicate relative molecular mass in kDa. Arrows in (*b*) indicate the positions of pp80, pp42 and pp37.

phoresis was performed with 7.8% (v/v) acetic acid/2.5% (v/v) formic acid, pH 1.8, in water at 1200 V for 2 h, and in the second dimension with 5% (v/v) acetic acid/0.5% (v/v) pyridine, pH 3.5, in water at 1100 V for 1 h. Phosphoamino acid markers were located with ninhydrin staining and radioactive spots by autoradiography.

RESULTS

The first trypanosomes to appear in the bloodstream of lethally irradiated laboratory rats, 4 days after infection with an inoculum of 10^4 *T. b. brucei* ILTat 1.1, are long and slender organisms with a free flagellum (Fig. 1*a*). At 7 days after infection more than 95% of the bloodstream trypanosomes are short and stumpy. They contain dark lipid granules and do not have a free flagellum (Fig. 1*b*). The course of infection of *T. b. brucei* ILTat 1.1 in lethally irradiated rats (Fig. 1*c*) shows that the logarithmic growth rate follows a 3 day pre-patent period until a maximum parasitaemia of 10^9/ml is attained. The percentage of long slender trypanosomes decreases from 100% on day 4 after infection to 5% or less by day 7. There is a corresponding increase in short stumpy form parasites from 0 to 95% or more during this period.

Although there is a great similarity between the Coomassie-Blue-stain protein profiles of trypanosomes isolated 4–7 days after infection (Fig. 2*a*, lanes 4–7), as the proportion of stumpy trypanosomes increases from 0 to 95%, a number of faint bands of approx. molecular mass 32–43 kDa appear (Fig. 2*a*, lanes 6 and 7). However, there are clear differences in the phosphoprotein profiles of endogenous cytosolic proteins from trypanosomes isolated 4–7 days after infection and phosphorylated *in vitro* (Fig. 2*b*). On differentiation from slender- to stumpy-form parasites there is a progressive decrease in phosphorylation of a 80 kDa protein (pp80; Fig. 2*b*, uppermost arrow), as well as an increase in phosphorylation of two proteins of 42 kDa (pp42) and 37 kDa (pp37) (Fig. 2*b*, second and third arrows respectively). Compared with slender parasites, there was a 3-fold increase of ^{32}P incorporated into pp42 and an 8-fold increase into pp37 in homogenates from stumpy trypanosomes. Endogenous protein phosphorylation of the total homogenate and of the insoluble and cytosolic fractions of the stumpy trypanosomes showed that both pp37 and pp42 are predominantly cytosolic proteins (results not shown).

Two-dimensional PAGE analysis showed that pp37 and pp42 consisted of multiple spots which focused at the acidic end of the gels (Fig. 3). Isoelectric focusing (results not shown) revealed

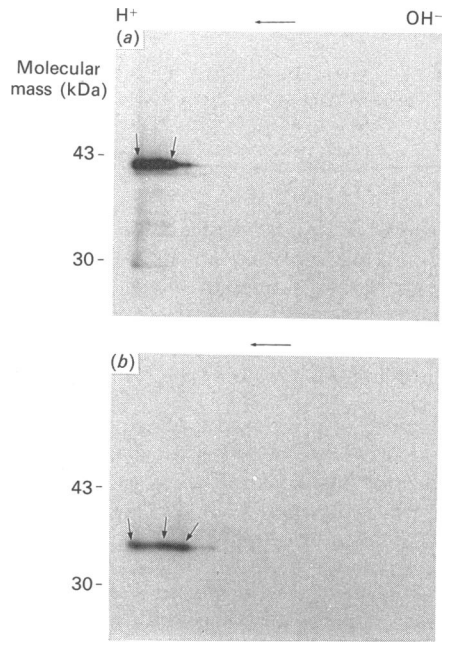

Fig. 3. Two-dimensional PAGE of electroeluted pp42 and pp37

^{32}P-labelled pp42 (*a*) and pp37 (*b*), electroeluted from polyacrylamide gels, were subjected to isoelectric focusing in polyacrylamide tube gels in the presence of urea, Nonidet P-40 and carrier ampholytes (pH 3.5 to 10), followed by electrophoresis in 10% (w/v) polyacrylamide/SDS slab gels and autoradiography. Small arrows indicate the several spots exhibited by each protein; the long arrow indicates the direction of electrofocusing.

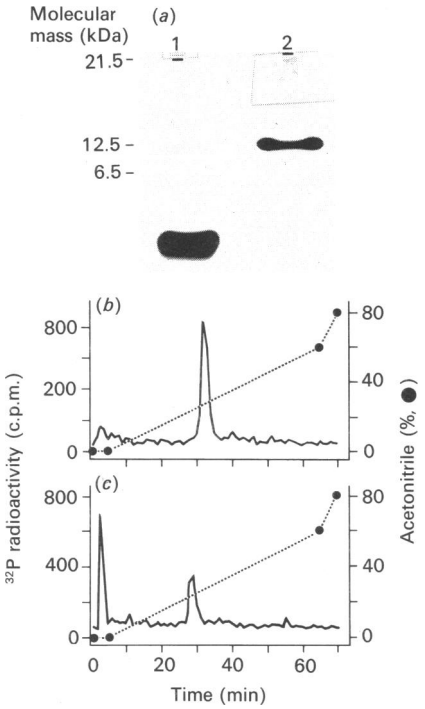

Fig. 4. Analysis of tryptic phosphopeptides of pp42 and pp37 by SDS/PAGE and h.p.l.c.

(*a*) ^{32}P-labelled protein bands corresponding to pp42 and pp37 were excised from SDS/PAGE gels and incubated for 18 h with Tos-Phe-CH_2Cl-treated trypsin in 100 mM-NH_4HCO_3, pH 7.9. The phosphopeptides derived by proteolysis were separated in a 10–20% (w/v) linear gradient polyacrylamide gel followed by autoradiography. Tryptic phosphopeptides of pp42 (lane 1) and pp37 (lane 2) are shown. The estimated molecular mass of pp42 tryptic peptide is < 5 kDa. (*b*) and (*c*) Electroeluted ^{32}P-labelled pp42 and pp37 were digested with Tos-Phe-CH_2Cl-treated trypsin and the phosphopeptides were separated on an Ultrasphere ODS column. Phosphopeptides were eluted with sequential linear gradients of acetonitrile: 0–60% over 60 min, followed by 60–80% over 5 min. Fractions were analysed by liquid scintillation counting. Elution profiles of pp42 peptides (*b*) and pp37 peptides (*c*) are shown.

that these proteins had pI values ranging between 3.2 and 4.5. It is likely that the multiple spots are a consequence of different extents of phosphorylation. Although the possibility that the different spots may be distinct proteins has not been excluded, peptide mapping in the case of pp42, which gave only a single product after trypsin treatment (see below), does not support this contention.

Exhaustive digestion of pp42 with trypsin produced a single phosphopeptide of less than 5 kDa (Fig. 4*a*, lane 1), whereas the tryptic phosphopeptide profile of pp37 (Fig. 4*a*, lane 2) revealed a single phosphopeptide of approx. 14 kDa. The elution profile of the tryptic phosphopeptides of pp42 and pp37 from an h.p.l.c. C_{18} reversed-phase column is shown in Fig. 4(*b*). Chromatography of the pp42 tryptic digest produced a single ^{32}P-labelled peptide which was eluted by 28% acetonitrile. In contrast, a similar fractionation of the tryptic digest of pp37 produced two ^{32}P-labelled peaks. The first peak was eluted at the solvent front, and the second was eluted at 23% acetonitrile. Analysis of the time course of pp42 digestion with trypsin gave no obvious intermediate product with a molecular mass corresponding to that of pp37. The failure to produce similar final proteolytic breakdown products, or even similar intermediates in the digestion process, suggests that it is unlikely that pp42 and pp37 are related proteins (e.g. by limited proteolysis of a larger protein to produce a smaller one).

Phosphoamino acid analysis on two-dimensional electrophoresis (results not shown) indicated that both proteins were phosphorylated mainly on serine residues, with some phosphorylation of threonine residues. Phosphotyrosine was not detected in either the acid or the alkaline hydrolysates.

A study of the conditions for the phosphorylation of pp42 and pp37 was conducted. Initially, the phosphatase inhibitors NaF, isobutylmethylxanthine and sodium orthovanadate were added to protein phosphorylation reactions. However, we observed that varying the concentrations of the three compounds from 0 to 5 mM had no effect on phosphorylation of the stumpy trypanosome cytosolic proteins (results not shown). The phosphatase inhibitors were therefore omitted from further phosphorylation reactions. Known effectors of protein phosphorylation, such as cyclic AMP and Ca^{2+} with either calmodulin or phospholipids, had no effect on the incorporation of phosphate into any of the cytosolic proteins of stumpy trypanosomes. Phosphorylation of pp42 occurred over a rather wide pH range from 6.5 to 9.0. Maximal phosphorylation of pp37 occurred in a somewhat narrower alkaline pH range (pH 8–9), although some phosphorylation could be seen in buffers with a pH as low as pH 6.0 (results not shown). Both GTP and ATP were acceptable phosphoryl donors. Indeed, 1.5–2-fold more [^{32}P]P_i was incor-

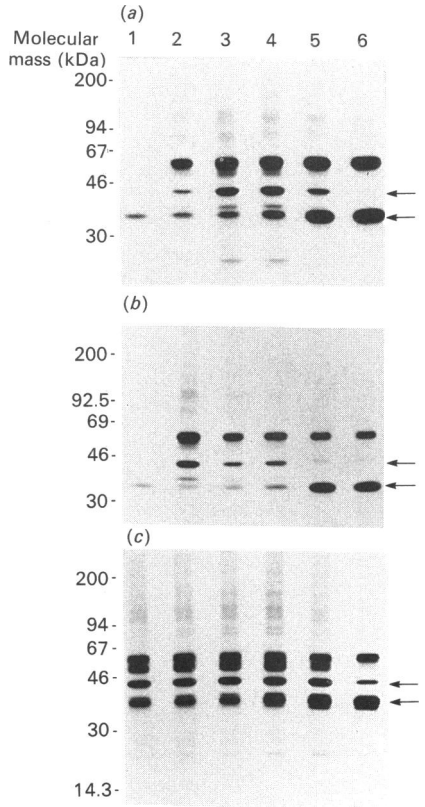

Fig. 5. Effects of metal ions on the phosphorylation of pp42 and pp37

Cytosolic extracts of stumpy trypanosomes were phosphorylated in the presence of increasing concentrations of $MgCl_2$ (*a*), $MnCl_2$ (*b*) or KCl (*c*) under standard assay conditions. ^{32}P-labelled proteins were resolved by SDS/PAGE and detected by autoradiography. Lanes 1–6 show phosphorylation performed in the presence of 0, 1, 5, 10, 50 and 100 mM-$MgCl_2$ (*a*) or -$MnCl_2$ (*b*) and 0, 10, 20, 50, 100 and 200 mM-KCl (*c*) respectively. Arrows show the positions of pp42 and pp37.

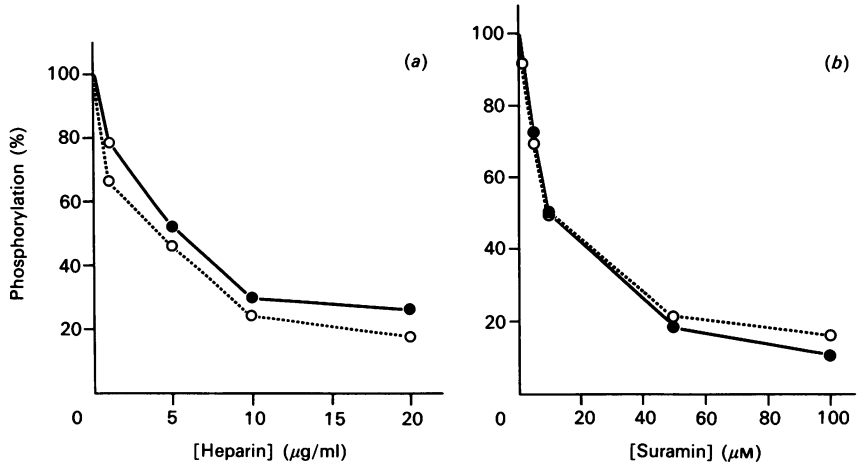

Fig. 6. Inhibition of phosphorylation of pp42 and pp37 by the polysulphated compounds heparin (*a*) and suramin (*b*)

Phosphorylation of cytosolic proteins was performed as described in the Materials and methods section with the addition of increasing concentrations of heparin or suramin. ^{32}P-labelled proteins, separated by SDS/PAGE, were detected by autoradiography and the amounts of phosphate incorporated into pp42 (○) and pp37 (●) were determined by liquid scintillation counting of the corresponding protein bands excised from the gels.

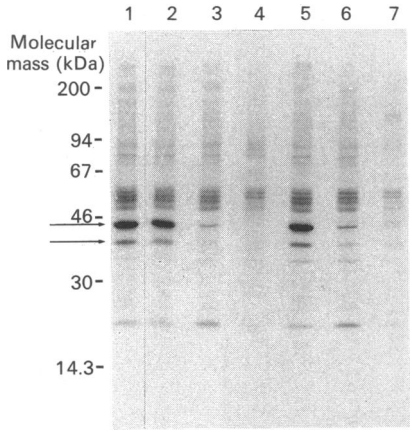

Fig. 7. Effects of polylysine and polyarginine on phosphorylation of cytosolic proteins in extracts of stumpy trypanosomes

Cytosolic extracts were phosphorylated under standard assay conditions in the presence of 1, 10 and 100 μg of polylysine/ml (lanes 2–4) or the same concentrations of polyarginine (lanes 5–7). Lane 1 shows the reaction performed without either effector. Phosphorylated proteins were analysed by SDS/PAGE and autoradiography. Arrows indicate pp42 and pp37.

porated in the presence of GTP than in the presence of ATP (results not shown).

The phosphorylation of pp42 and pp37 was found to be dependent on the presence of Mg^{2+} or Mn^{2+}. Maximum phosphorylation of pp42 was attained with the addition of either 10 mM-Mg^{2+} or 1 mM-Mn^{2+}. Higher concentrations of each cation became inhibitory. However, the concentration of Mg^{2+} required for the optimum phosphorylation of pp37 was in excess of 100 mM (Fig. 5*a*), while that for Mn^{2+} was 50 mM (Fig. 5*b*). The phosphorylations of pp42 and pp37 were also affected differently by KCl. The phosphorylation of pp37 was increased dramatically by the addition of KCl (200 mM-KCl increased phosphorylation approx. 4-fold), but the effect of KCl on the phosphorylation of pp42 was more ambiguous. Concentrations of KCl up to 100 mM had a slight activatory effect, but higher concentrations were inhibitory (Fig. 5*c*).

The negatively charged glycosaminoglycan heparin inhibited, in a dose-dependent manner, the incorporation of phosphate into all phosphorylatable *T. brucei* proteins, including pp42 and pp37; 50% inhibition of phosphorylation of pp42 and pp37 occurred at approx. 5 μg of heparin/ml (Fig. 6*a*). Suramin, an anti-trypanosomal naphthylamine, also inhibited phosphorylation of all proteins in a dose-dependent manner (Fig. 6*b*); 50% inhibition of pp42 and pp37 phosphorylation occurred with 10 μM-suramin. However, positively charged polybasic peptides such as polylysine and polyarginine also inhibited phosphorylation of pp42 and pp37, although they had relatively little effect on the phosphorylation of other proteins (Fig. 7). Polyamines also inhibited phosphorylation of pp42 and pp37 (Figs. 8*a* and 8*b* respectively) in the order spermine > spermidine > putrescine. For example, 50% inhibition of pp37 was achieved with 2.5 mM-spermine and 5 mM-spermidine (see Fig. 8*b*). However, 50% inhibition was not achieved with putrescine concentrations of up to 10 mM (Figs. 8*a* and 8*b*).

The above results suggest that the phosphorylation of pp42 and pp37 is catalysed by unusual casein-kinase-like enzymes. The presence of such casein-kinase-like enzymes in trypanosomes is clearly shown by the ability of the trypanosome lysates to phosphorylate such classic substrates as casein and phosvitin (Figs. 9*a* and 9*b* respectively). Interestingly, the slender form trypanosomes have three times more casein-kinase-like activity than the stumpy parasites. Thus the differences in phosphorylation observed with pp42 and pp37 are probably a consequence of differences in the availability of phosphorylatable substrates *in vitro*, not of enhanced enzyme levels, in the stumpy trypanosomes. To test this hypothesis, heat-inactivated cytosolic extracts were used as substrates in phosphorylation reactions. Both slender and stumpy trypanosome extracts were able to phosphorylate pp42. However, pp42 appeared to be present only in the stumpy trypanosome extracts (Fig. 9*d*, lanes 3 and 4). The results also show the presence of an 80 kDa protein in the cytosolic extracts of slender trypanosomes, but not in the extracts of stumpy forms, which are phosphorylated by enzyme(s) from both slender and stumpy parasites (Figs. 9*c* and 9*d*). No phosphorylation of a protein equivalent to pp37 was observed in these heat-treated extracts, perhaps because of heat-induced denaturation.

It has been reported that the trypanocidal compound DFMO

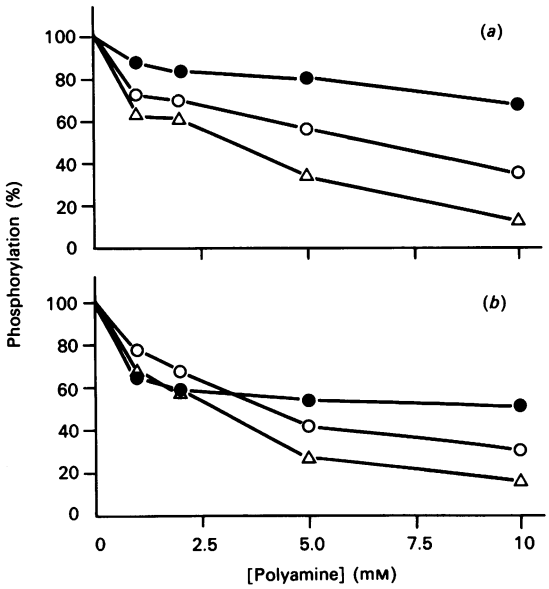

Fig. 8. Effect of polyamines on the phosphorylation of pp42 and pp37

Cytosolic proteins from stumpy trypanosomes were phosphorylated under standard assay conditions in the presence of various concentrations of putrescine, spermidine or spermine. Phosphorylated proteins were separated by SDS/PAGE and detected by autoradiography. Phosphate incorporation into pp42 and pp37 was determined by liquid scintillation spectrometry of the corresponding protein bands excised from the gels. The amount incorporated into pp42 (*a*) and pp37 (*b*) was determined as a function of the concentration of putrescine (●), spermidine (○) and spermine (△).

induces a transformation of long slender trypanosomes into short stumpy organisms (Garofalo *et al.*, 1982; Bacchi *et al.*, 1983). We therefore tested the effect of this compound on the induction of phosphorylation of pp42 and pp37. Trypanosomes were isolated from infected rats provided with drinking water containing 2% DFMO (Bacchi *et al.*, 1983). After 24 h of treatment with DFMO there was a 2-fold increase in phosphorylation of pp42 and a 4-fold increase in pp37 phosphorylation compared with controls (Fig. 10). There was also a decrease in the phosphorylation of an 80 kDa protein in trypanosomes isolated from DFMO-treated animals. The phosphorylation of this protein disappeared altogether with differentiation of long slender trypanosomes into short stumpy forms (Fig. 10, lanes 1 and 5 respectively).

DISCUSSION

The data presented in this paper have identified phosphorylatable proteins which can serve as markers for differentiation in *T. b. brucei*. There is a coincidental increase in the phosphorylation of two acidic proteins (pp42 and pp37) *in vitro* after the differentiation of bloodstream forms of *T. b. brucei* from rapidly dividing long slender parasites into non-dividing short stumpy parasites. At the same time, there is a decrease in phosphorylation of an 80 kDa protein. Other experiments showed that the amount of pp42 is lowered not only in the long slender trypanosomes, but also in the procyclic (tsetse midgut form) trypanosomes (T. Aboagye-Kwarteng, unpublished work). Since both the long slender and the procyclic trypanosomes are dividing cells, whereas short stumpy forms are not, this suggests that phosphorylation of pp42 and pp37 may be specific to the non-dividing stage. It will therefore be of interest to determine whether similar changes

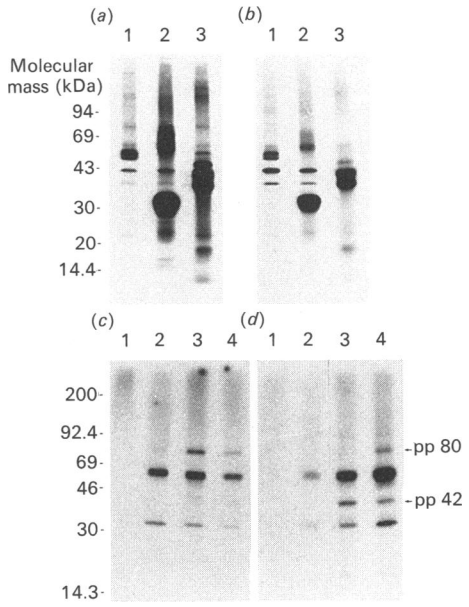

Fig. 9. Phosphorylation of endogenous and exogenous protein substrates by long slender and short stumpy trypanosome protein kinases

Casein, phosvitin (1 mg/ml) or heat-denatured trypanosome cytosolic proteins were incubated with the cytosolic extracts of slender or stumpy parasites. Proteins were resolved by SDS/PAGE on a 7.5–15% (w/v) linear gradient polyacrylamide gel and phosphoproteins were identified by autoradiography. (*a*) Phosphorylation of casein (lane 2) and phosvitin (lane 3) by cytosolic extracts of long slender forms; (*b*) phosphorylation of casein (lane 2) and phosvitin (lane 3) by cytosolic extracts of short stumpy forms. Lane 1 in (*a*) and (*b*) contained cytosolic extracts of long slender and short stumpy trypanosomes respectively, without exogenous substrate. (*c*) Studies with heat-denatured cytosolic extracts of long slender trypanosomes used as substrates: lane 1, substrate alone; lane 2, 1 μg of undenatured cytosolic extract of slender trypanosomes alone; lane 3, 1 μg of undenatured cytosolic extract of slender trypanosomes with heat-denatured substrate; lane 4, 1 μg of undenatured cytosolic extract of stumpy trypanosomes with substrate. (*d*) Studies with heat-denatured cytosolic extract of short stumpy trypanosomes used as substrate: lane 1, substrate alone; lane 2, 1 μg of cytosolic extract of stumpy forms alone; 3, 1 μg of cytosolic extract of stumpy forms with substrate; lane 4, 1 μg of cytosolic extract of slender trypanosomes with substrates. Arrows indicate the positions of pp80 and pp42; pp37 was not observed.

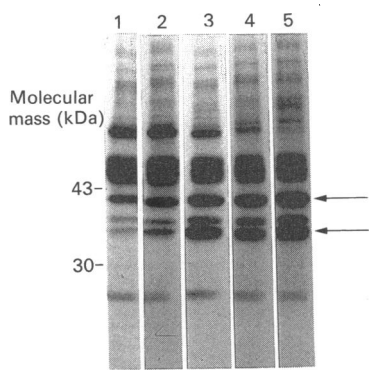

Fig. 10. Phosphoprotein profiles of trypanosomes isolated from untreated and DFMO-treated rats

Cytosolic extracts of trypanosomes recovered from untreated and DFMO-treated rats were prepared as described in the Materials and methods section. Proteins were phosphorylated under standard assay conditions and resolved by SDS/PAGE, and phosphorylated proteins were identified by autoradiography. Shown are trypanosomes from untreated rats: day 4 (lane 1), day 5 (lane 2) and day 7 (lane 5); trypanosomes from rats treated with DFMO for 24 h (lane 3); trypanosomes from rats treated with DFMO for 48 h (lane 4). Arrows indicate pp42 and pp37.

in phosphorylation occur in other dividing and non-dividing life cycle stages of *T. b. brucei*, as well as in other trypanosomes such as *T. vivax* and *T. congolense* that do not exhibit such striking morphological changes.

Since the cytosolic extracts used for the routine labelling of pp42 and pp37 *in vitro* were a source of both substrate and enzyme, it was of interest to determine whether the differential phosphorylation of pp42 and pp37 was due to changes in substrate, enzyme or other factors in the respective extracts. To investigate this, catalytic amounts of slender and stumpy trypanosome cytosolic extracts were added to heat-inactivated trypanosomal extracts as well as casein and phosvitin. Cytosolic extracts of both slender and stumpy trypanosomes were capable of phosphorylating the acidic proteins casein and phosvitin. The extracts of slender trypanosomes phosphorylated casein and phosvitin with a greater efficiency than those of stumpy trypanosomes. The phosphorylation of pp42, which occurred only in heat-inactivated cytosolic extracts of stumpy trypanosomes, was catalysed by both slender and stumpy trypanosome enzyme sources. Thus the differential phosphorylation of pp42 appears to be due to differences in substrate rather than enzyme(s). The disappearance of the 80 kDa protein from the stumpy extracts also appears to be a consequence of altered levels of substrate rather than enzyme. The possibility cannot be discounted that the differential phosphorylation of these proteins *in vitro* may be due to different levels of phosphorylation *in vivo*. However, preliminary studies with antiserum to pp37 by Western blot analysis suggest that there are increased amounts of pp37 in the stumpy parasites (T. Aboagye-Kwarteng, unpublished work). Moreover, the Coomassie Blue-stained protein profiles of trypanosomes isolated at 4–7 days post-infection (Fig. 2*a*) show the appearance of a number of bands, two of which co-migrate with pp37 and pp42, in the stumpy trypanosomes. The possibility that the Coomassie Blue-stained bands correspond to pp37 and pp42 strengthens the suggestion that the two phosphorylatable proteins are induced when trypanosomes differentiate from slender to stumpy parasites.

The phosphorylation of pp42 and pp37 occurs on serine and threonine residues and appears to be catalysed by an unusual type of casein kinase. Several major classes of protein kinases were eliminated from consideration, as effectors such as cyclic AMP, cyclic GMP, Ca^{2+}/calmodulin and Ca^{2+}/phospholipid had no effect on phosphorylation of either of the phosphoproteins, or indeed of any cytosolic proteins of stumpy trypanosomes. The ready phosphorylation of pp42 and pp37 using either GTP or ATP as phosphoryl donors and the inhibition of this reaction by heparin suggest that the phosphorylating activity is casein-kinase-like (Hathaway & Traugh, 1982; Hathaway *et al.*, 1983).

The activating effects of Mg^{2+}, Mn^{2+} and KCl, and the broad pH range for the phosphorylation of pp42 and pp37, suggest that trypanosome cytosolic protein kinases may belong to the casein kinase II class of enzymes (Hathaway & Traugh, 1982). However, the phosphorylation of pp42 and pp37, as well as that of the exogenous acidic substrates casein and phosvitin, was inhibited by positively charged molecules such as polyarginine, polylysine and a variety of polyamines. These cations are all activators of mammalian casein kinase II enzymes (Tuazon *et al.*, 1979; Hathaway *et al.*, 1983; Meggio & Pinna, 1984; Bar-Zvi & Branton, 1986; Kishimoto *et al.*, 1987). Thus the activities which phosphorylate pp42 and pp37 are similar to, but distinct from, mammalian casein kinase II enzymes.

Maximum phosphorylation was obtained with Mg^{2+} concentrations in excess of the amount required for formation of a Mg–ATP complex, suggesting that the phosphorylation of pp42 and pp37 is activated directly by Mg^{2+}. Whether this enhancement of phosphorylation occurs through activation of distinct enzymes, as has been reported for mammalian casein kinases (Hathaway & Traugh, 1984; Plana *et al.*, 1985), or by a change in the conformation of the substrates by binding Mg^{2+}, or by a combination of these effects, is not yet clear. However, the observation that different concentrations of these metal ions were required for optimal phosphorylation of pp42 and pp37 indicates that the metal ion activation of this phosphorylation process is a complex event.

Heparin, a polysulphated glycosaminoglycan, inhibits the phosphorylation of pp42 and pp37 in *T. brucei* as well as that of a variety of proteins in *Leishmania donovani*. The latter parasite, a related protozoon, not only possesses heparin receptors on its surface but is also able to incorporate [^{35}S]sulphate into a cell-associated macromolecule with the properties of heparin proteoglycan (Mukhopadhyay *et al.*, 1989). Thus the possibility exists that heparin-like macromolecules represent a class of endogenous inhibitors of kinetoplastid protein kinases. Like heparin, the trypanocidal polysulphated naphthylamine, suramin, inhibits the phosphorylation of pp42 and pp37. Suramin also induces morphological changes in *T. brucei* which resemble the slender-to-stumpy form trypanosome differentiation (T. Aboagye-Kwarteng, unpublished work). Moreover, suramin induces differentiation in transformed neuroblastoma cells and blocks phosphorylation in these cells by inhibiting protein kinase C (Hensey *et al.*, 1989). However, the latter authors did not report whether there was any effect of suramin on casein kinases in their neuroblastoma cell lines.

Phosphorylation of pp42 and pp37 was inhibited by putrescine, spermidine and spermine in addition to heparin and suramin. This is quite unusual, because polyamines have been shown to activate mammalian casein kinases (Hathaway & Traugh, 1982). Inhibition of protein kinase activity by polyamines has also been observed for *T. cruzi* (Walter & Ebert, 1979), and may thus be an unusual property of trypanosomatid protein kinases. These compounds (putrescine, spermidine, spermine), which have been shown to be present in most prokaryotic and eukaryotic cells in millimolar amounts, have been implicated in regulation of such biological phenomena as cell growth and differentiation (Tabor & Tabor, 1984; Pegg, 1986). The first enzyme in the biosynthetic pathway of polyamines, ornithine decarboxylase, has been shown to be phosphorylated by a polyamine-dependent protein kinase (Atmar & Kuehn, 1981) as well as by casein kinase II (Meggio *et al.*, 1984). The phosphorylation of ornithine decarboxylase sharply inhibits its decarboxylating activity. The trypanocidal drug DFMO, a potent inhibitor of trypanosome ornithine decarboxylase (Garofalo *et al.*, 1982), causes a rapid depletion of cellular polyamines, a decline of RNA and DNA synthesis, an apparent blockage of cytokinesis and an induction of morphological changes resembling the long-slender-to-short-stumpy differentiation (Bacchi *et al.*, 1983). We have shown that, in comparison with that of untreated trypanosomes, the phosphoprotein profiles of DFMO-treated trypanosomes exhibited an increased phosphorylation of pp42 and pp37 similar to the phosphorylation changes observed in the slender-to-stumpy conversion. These results suggest a possible role for protein phosphorylation in the regulation of cell growth and differentiation in *T. b. brucei*. By analogy with the phosphorylation changes that occur with $p34^{cdc2}$ in the cell cycle of other eukaryotic cells, the identification of these differentially phosphorylatable proteins provides a means for studying biochemical changes which occur between dividing and non-dividing trypanosomes.

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REFERENCES

Aboagye-Kwarteng, T. (1983) M.Sc. Thesis, University of Ghana

Arion, D., Meijer, L., Brizuela, L. & Beach, D. (1988) Cell **55**, 371–378

Atmar, V. J. & Kuehn, G. D. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 5518–5522

Bacchi, C. J., Garofalo, J., Mockenhaupt, D., McCann, P. P., Diekema, K. A., Pegg, A. E., Nathan, H. C., Mullaney, E. A., Chunosoff, L., Sjoerdsma, A. & Hutner, H. S. (1983) Mol. Biol. Parasitol. **7**, 209–225

Bar-Zvi, D. & Branton, D. (1986) J. Biol. Chem. **261**, 9614–9621

Beach, D. H., Durkacz, B. & Nurse, P. M. (1982) Nature (London) **300**, 706–709

Bowman, I. B. R. & Flynn, I. W. (1976) in Biology of the Kinetoplastida (Lumsden, W. H. R. & Evans, D. A., eds.), vol. 1, pp. 435–476, Academic Press, London

Cohen, P. (1982) Nature (London) **296**, 613–620

Collett, M. S., Purchio, A. F. & Erikson, R. L. (1980) Nature (London) **285**, 167–169

Cooper, J. A., Sefton, B. M. & Hunter, T. (1983) Methods Enzymol. **99**, 387–405

Draetta, G. & Beach, D. (1988) Cell **54**, 17–26

Draetta, G., Brizuela, L., Potashkin, J. & Beach, D. (1987) Cell **50**, 319–325

Draetta, G., Beach, D. & Moran, E. (1988) Oncogene **2**, 553–557

Dunphy, W. G., Brizuela, L., Beach, D. & Newport, J. (1988) Cell **54**, 423–431

Edelman, A. M., Blumenthal, D. K. & Krebs, E. G. (1987) Annu. Rev. Biochem. **56**, 567–613

Ek, B., Westermark, B., Wasteson, A. & Heldin, C. H. (1982) Nature (London) **295**, 419–420

Fairlamb, A. H. & Opperdoes, F. R. (1986) in Carbohydrate Metabolism in Cultured Cells (Morgan, M. J., ed.), pp. 183–225, Plenum Publishing Corp., New York

Flynn, I. W. & Bowman, I. B. R. (1973) Comp. Biochem. Physiol. B **45**, 24–42

Garofalo, J., Bacchi, C. J., McLaughlin, S. D., Mockenhaupt, D., Trueba, G. & Hutner, S. H. (1982) J. Protozool. **29**, 389–394

Gautier, J., Norbury, C., Lohka, M., Nurse, P. & Maller, J. (1988) Cell **54**, 433–439

Grab, D. J. & Bwayo, J. J. (1982) Acta Trop. **39**, 363–366

Hathaway, G. M. & Traugh, J. A. (1982) Curr. Top. Cell. Regul. **21**, 101–127

Hathaway, G. M. & Traugh, J. A. (1984) Arch. Biochem. Biophys. **233**, 133–138

Hathaway, G. M., Tuazon, P. T. & Traugh, J. A. (1983) Methods Enzymol. **99**, 308–317

Hensey, C. E., Boscoboinik, D. & Azzi, A. (1989) FEBS Lett. **258**, 156–158

Hunter, T. & Cooper, J. A. (1981) Cell **24**, 741–752

Hunter, T. & Cooper, J. A. (1985) Annu. Rev. Biochem. **54**, 897–930

Hunter, T. & Sefton, B. M. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 1311–1315

Kasuga, M., Zick, Y., Blithe, D. L., Crettaz, M. & Kahn, C. R. (1982) Nature (London) **298**, 667–669

Kishimoto, A., Brown, M. S., Slaughter, C. A. & Goldstein, J. L. (1987) J. Biol. Chem. **262**, 1344–1351

Krebs, E. G. (1985) Biochem. Soc. Trans. **13**, 813–820

Labbe, J., Lee, M., Nurse, P., Picard, A. & Doree, M. (1988) Nature (London) **335**, 251–254

Laemmli, U. K. (1970) Nature (London) **227**, 680–685

Lanham, S. M. & Godfrey, D. G. (1970) Exp. Parasitol. **28**, 521–531

Lee, M. G. & Nurse, P. (1986) Trends Genet. **4**, 287–290

Lee, M. G. & Nurse, P. (1987) Nature (London) **327**, 31–35

Lee, M. G., Norbury, C. J., Spurr, N. K. & Nurse, P. (1988) Nature (London) **333**, 676–679

Manai, M. & Cozzone, A. J. (1982) Anal. Biochem. **124**, 12–18

Martensen, T. M. (1984) Methods Enzymol. **107**, 3–23

Mbawa, Z. R., Gumm, I. D., Fish, W. R. & Lonsdale-Eccles, J. D. (1991) Eur. J. Biochem., in the press

Meggio, F. & Pinna, L. A. (1984) Eur. J. Biochem. **145**, 593–599

Meggio, F., Flamigni, F., Calderera, C. M., Gaurnier, C. & Pinna, L. A. (1984) Biochem. Biophys. Res. Commun. **122**, 997–1004

Mukhopadhyay, N. K., Shome, K., Saha, A. K., Hassell, J. R. & Glew, R. H. (1989) Biochem. J. **264**, 517–525

Nairn, A. C., Hemmings, H. C., Jr. & Greengard, P. (1985) Annu. Rev. Biochem. **54**, 931–976

Nurse, P. & Bisset, Y. (1981) Nature (London) **292**, 558–560

Nurse, P. & Thuriaux, P. (1980) Genetics **96**, 627–637

O'Farrell, P. H. (1975) J. Biol. Chem. **250**, 4007–4021

Ormerod, W. E., Healey, P. & Armitage, P. (1963) Exp. Parasitol. **13**, 386–394

Pegg, A. E. (1986) Biochem. J. **234**, 249–262

Plana, M., Guash, M. D. & Itarte, E. (1985) Biochem. J. **230**, 69–74

Shapiro, S. Z., Naessens, J., Liesegang, B., Moloo, S. K. & Magondu, J. (1984) Acta Trop. **41**, 313–323

Shenolikar, S. (1988) FASEB J. **2**, 2753–2764

Simaris, V. & Nurse, P. (1986) Cell **45**, 261–268

Tabor, C. E. & Tabor, H. (1984) Annu. Rev. Biochem. **53**, 749–790

Tuazon, P. T., Bingham, E. W. & Traugh, J. A. (1979) Eur. J. Biochem. **94**, 497–504

Ui, N. (1971) Biochim. Biophys. Acta **229**, 567–581

Vickerman, K. (1965) Nature (London) **208**, 762–766

Vickerman, K. (1970) in African Trypanosomiases (Mulligan, H. W., ed.), pp. 60–66, Allen and Unwin, London

Vickerman, K. (1985) Br. Med. Bull. **41**, 105–114

Walter, R. D. & Ebert, F. (1979) Tropenmed. Parasitol. **30**, 9–12

White, M. F. & Kahn, C. R. (1986) Enzymes 3rd Ed. **17**, 247–310

Wijers, D. J. B. & Willett, K. C. (1960) Ann. Trop. Med. Parasitol. **54**, 341–346

Young, J. R. (1985) Ph.D. Thesis, University of Cambridge

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